Pre-Validation of the Aromatase Assay using Human and Bovine Placental, and Human Recombinant Microsomes

Endocrine Disruptor Methods Validation Subcommittee (EDMVS)
Plenary Session

June 5, 2003

turning knowledge into practice

Research Triangle Park, North Carolina

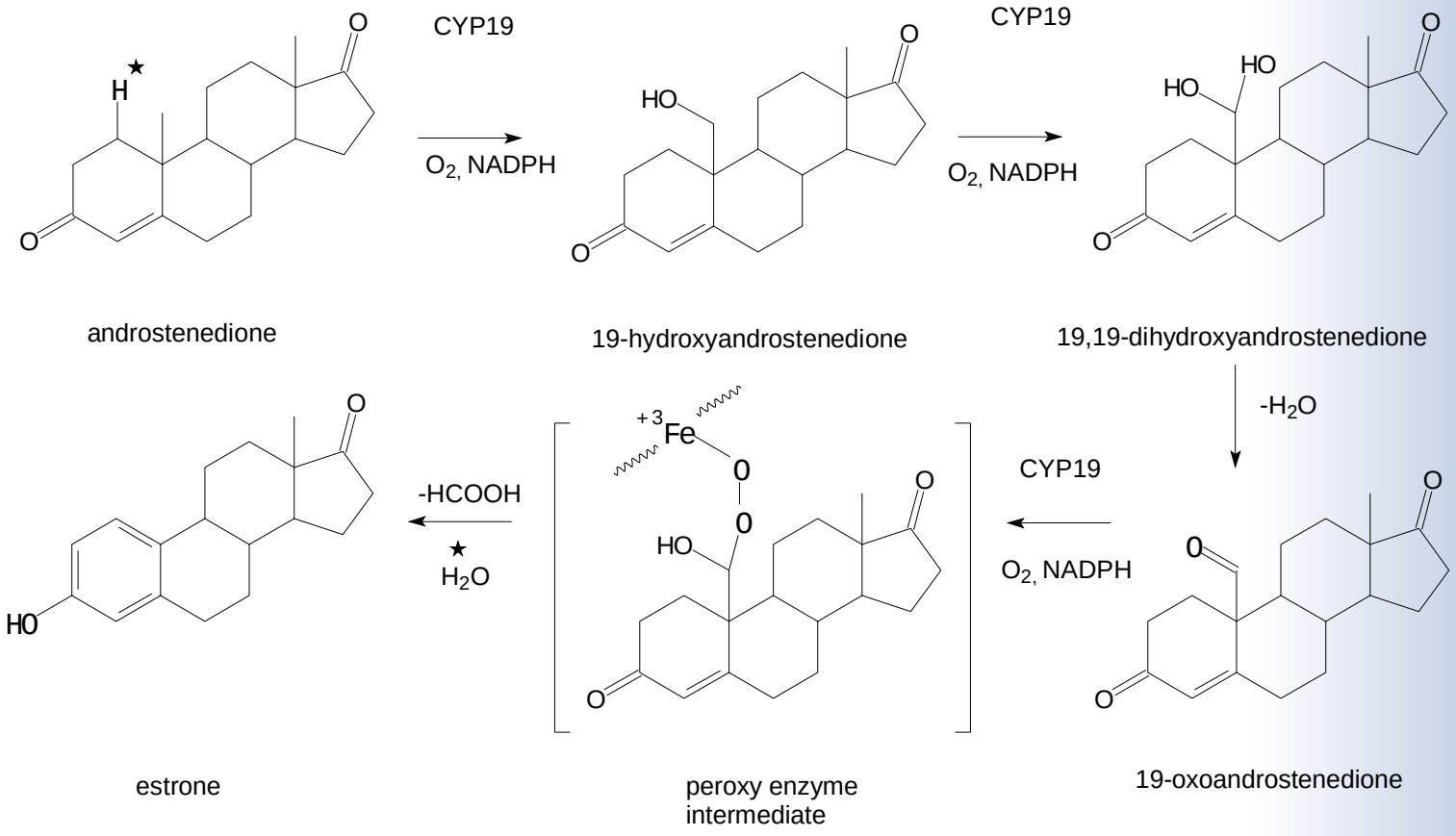
Overview

- Background: Aromatase (CYP19)
- Study Goals
- Substrate characterization
- Placenta tissues – human, bovine, porcine
- Methods
- Results
    - Protein yield
    - P450 Spectra
    - Aromatase activities
- Conclusions
- Future studies

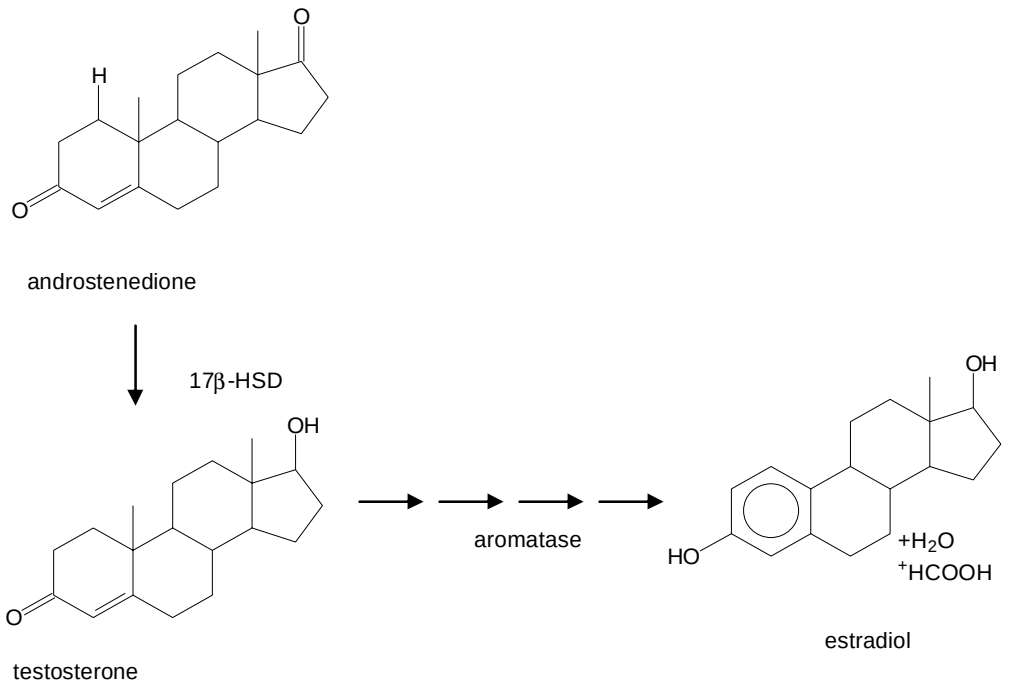
Aromatase

- Cytochrome P450 enzyme – CYP19
- Present in the gonads and placenta
- Responsible for the biosynthesis of estrogen steroid hormones
- Can be inhibited at the level of gene expression (e.g., ethylhexylphthalate), or directly at enzyme (e.g. azoles)

Steroid Hormone Biosynthesis by Aromatase



Steroid Hormone Biosynthesis by Aromatase (cont'd)



Study Goals

The goal of this work is to identify the optimal factors and conditions for the assay of aromatase, including:

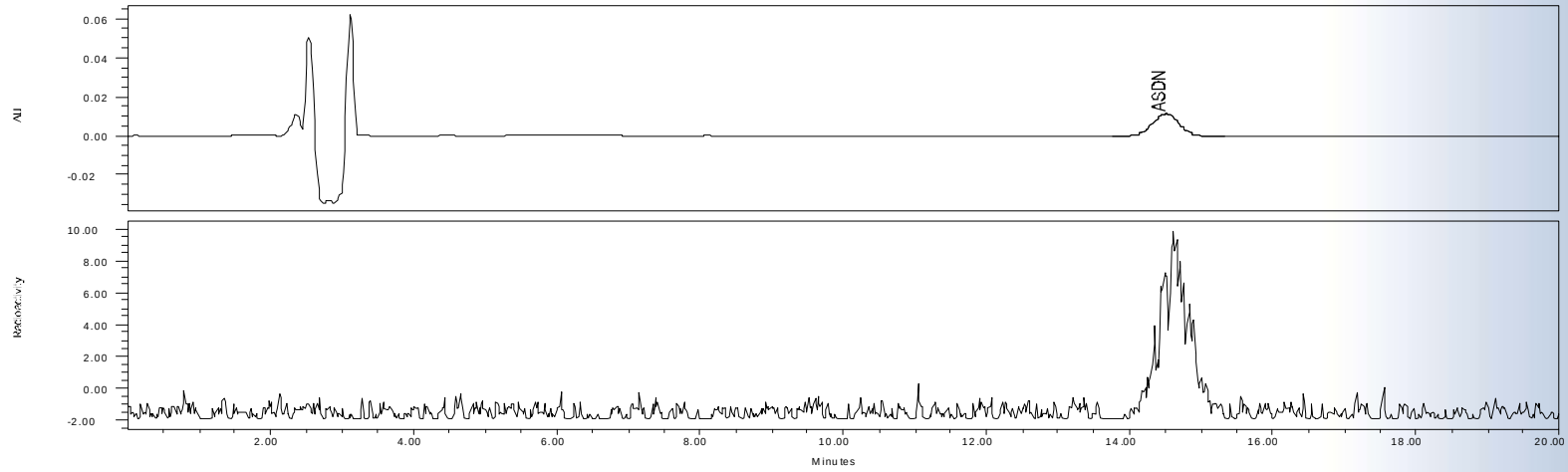
- Characterization of radiolabeled substrate (androstenedione)
- Selection of mammalian placenta allowing sufficient yield of catalytically-active microsomal protein, and assessment of human recombinant CYP19
- Optimization of assay with respect to concentration of protein, cofactors, substrate, and incubation time using a factorial design
- Using this optimized assay, determine the effect of selected substances on aromatase activity

Substrate Characterization

Sources

- Nonradiolabeled 4-androstene-3,17-dione (99%): Sigma Chemical Co.
- Radiolabeled [1β-^{3}H(N)]androst-4-ene-3,17-dione: Perkin-Elmer Life Science
- Specific activity: 25.3 Ci/mmol
- Radiochemical purity: 98%

HPLC Radiochromatogram for [^{3}H]Androstenedione



SampleName 1:1 10691-43A: 41C; Vial 5; Injection 1; Channel 2487Channel 1; Date Acquired 2/19/03 4:36:08 PM
SampleName 1:1 10691-43A: 41C; Vial 5; Injection 1; Channel SATIN ; Date Acquired 2/19/03 4:36:08 PM

Peak Results

	Name	RT	Area	Height
1	ASDN	14.506	293714	11441
2	[3H]ASDN	14.625	337219	11235

Column: Zorbax SB-C18, USCL011903, 250 x 4.6 mm
Mobile Phase: 55:15:30 ddH2O: THF:MeOH 10691-95A
Flow Rate: 1 mL/min
Detectors: Waters 2487 at 240 nm
B-RAM with 250 ul LiGL solid cell, #11590

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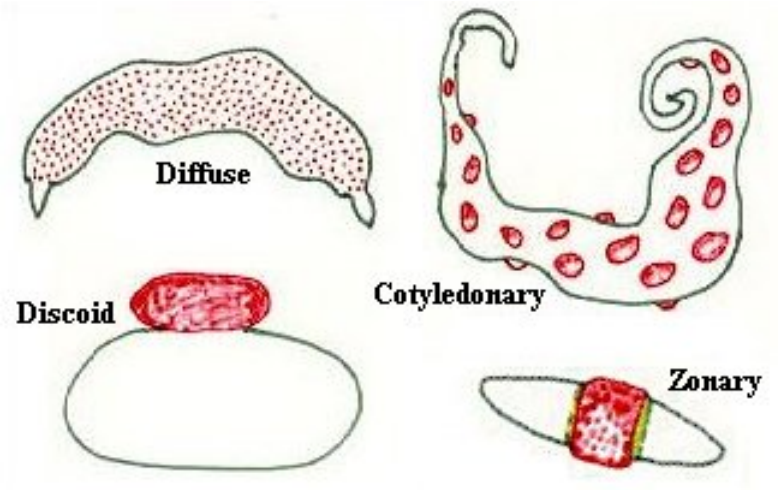
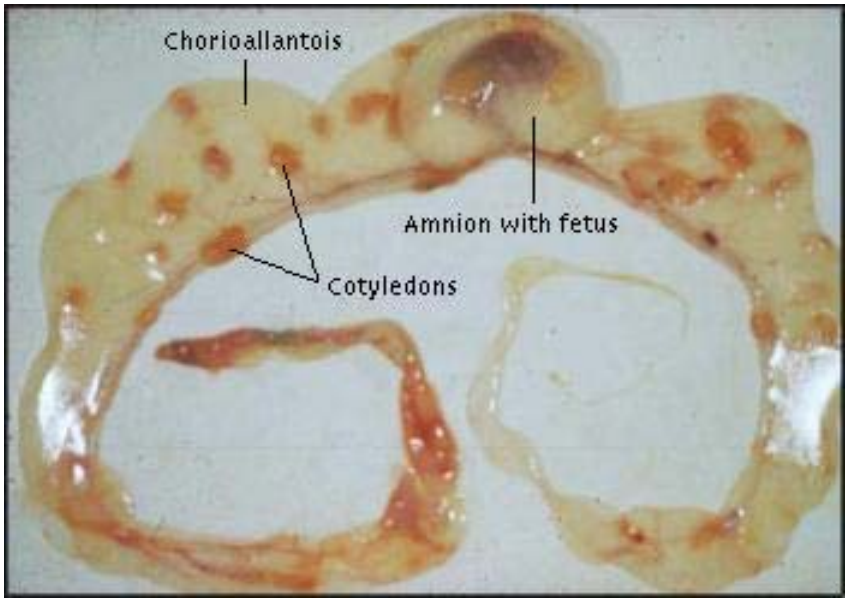
Placental Tissues



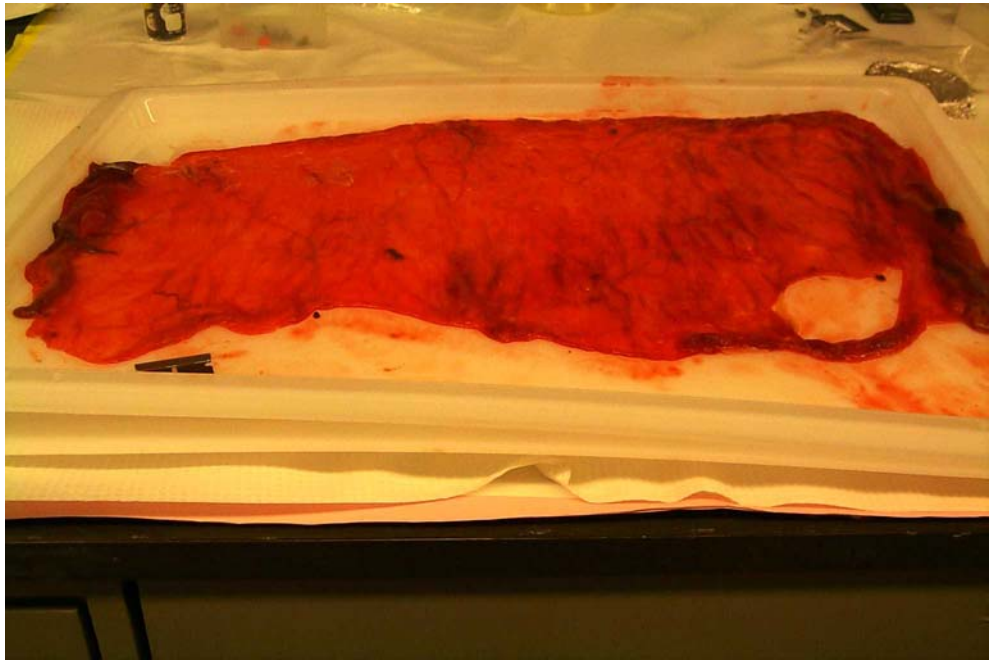
http://www.vivo.colostate.edu/hbooks/pathphys/reprod/placenta/structure.html

Placental Tissues (con't)



http://www.vivo.colostate.edu/hbooks/pathphys/reprod/placenta/ruminants.html

Placenta Tissues (con't)



Methods

Preparation of microsomes from tissues

- Iced-down (not frozen!) within 10 min of delivery
- Soft tissue harvested, homogenized in cold buffer
- Homogenate centrifuged @ 10,000*g* for 30 min, 4°C
- Supernatant centrifuged @ 100,000*g* for 60 min, 4°C
- Pellet resuspended in buffer, centrifuged @ 100,000*g* for 60 min, 4°C
- Pellet "washed" by repeating above
- Resuspended in buffer, protein concentration determined

Spectral P450 content determination: difference spectrum, 400 – 500 nm, of CO vs. CO/dithionite reduced microsomes, quantitation using extinction coefficient for the 450 nm absorbance of 100 mM^{-1} cm^{-1}

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Methods (cont'd)

Addition of the following into 16 x 100 mm test tubes:

0.1 mL	propylene glycol
50.0 nM	^{3}H-androstenedione (0.1 µCi)
1.7 mM	$NADP^{+}$
2.8 mM	glucose-6-phosphate
1.0 u	glucose-6-phosphate dehydrogenase

Dilute to 1.0 mL volume with phosphate buffer, pH 7.4, and warm to 37 °C

Preparation of placental or recombinant microsomal pellet

↓

Suspension of placental or recombinant microsomes and dilution to

~0.1 mg/mL in phosphate buffer

↓

Warm microsomes to 37 °C

↘ ↙

Add 1.0 mL placental microsomal suspension to each test tube

↓

Incubate at 37 °C for 30 minutes in shaking water bath

↓

Methods (cont'd)

Add 2.0 mL of CH_2Cl_2 to quench enzyme reaction; vortex for 30 seconds; centrifuge for 10 minutes at 500 rpm

↓

Transfer organic layer to a capped vial.

Add 2.0 mL of CH_2Cl_2 to test tube containing aqueous layer; vortex for 30 seconds; centrifuge for 10 minutes at 500 rpm

↓

Transfer organic layer to a capped vial.

Add 2.0 mL of CH_2Cl_2 to test tube containing aqueous layer; vortex for 30 seconds; centrifuge for 10 minutes at 500 rpm

↓

Transfer organic layer to a capped vial. Prepare aliquots of each organic extract for analysis by LSC.

Transfer aqueous layer to a capped vial; transfer 0.5 mL to

a LSC vial; add 10.0 mL scintillation cocktail; count in LSC.

Tissue Procurement Issues

Human placenta

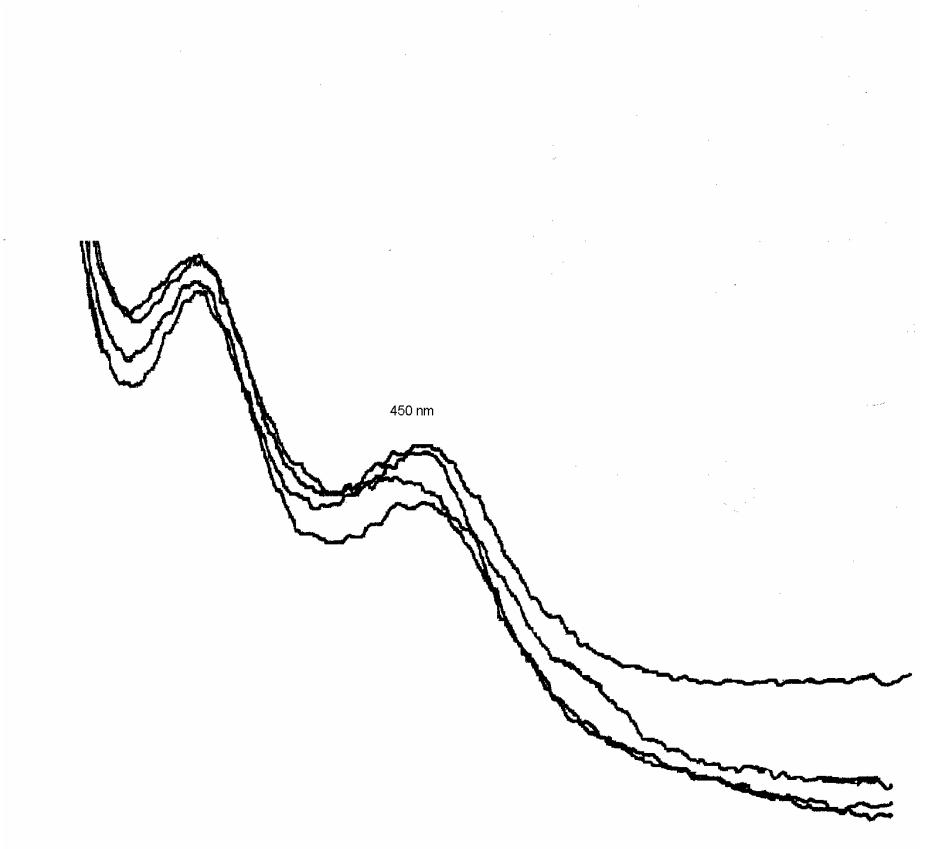
- Caesarian section allows for 1) timed delivery and optimal collection conditions, and 2) less chance of disease transmission

Bovine and porcine

- Requires farms close to laboratories, assistance of farm staff
- Deliveries seasonal, and any time of day

P450 Difference Spectrum

Human Recombinant CYP19



Results (cont'd)

Preoptimization Results

Enzyme Source	Microsomal protein yield (mg/g wet tissue processed)	P450 Content (nmol/mg protein)	Aromatase Activity (nmol/mg • min)
Human placenta	900/511.79 (1.76 mg/g)	0.048	0.015[a]
Human Recombinant	--	0.38	0.022
Bovine placenta	675/748.99 (0.9 mg/g)	0.031	0.003[b]
Porcine placenta*	126/257.44 (0.49 mg/g)	0.053	0.003

*Only 1 of 5 porcine placentas yielded microsomes with acceptable aromatase activity

[a]Acceptance criteria: 0.005 nmol product/mg protein/min

[b]Literature value: 0.0036 ± 0.00078 nmol estrone formed/mg protein/min (Tsumagari et al. (1993). *J. Reprod. Fert.* 1993, ***98***, 631-36.)

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Conclusions

Collection conditions used for placentas are crucial to activity

Human Placentas

- easiest to collect under optimal conditions
- well-defined morphology and good yield of microsomal protein
- high activity
- Human recombinant, comparable activity with best placental preparations

However,

- SOPs must be in place for handing potentially infectious materials
- Although Caesarian vs. birth canal delivery minimizes infection of the placental tissue, information regarding screening for HIV, hepatitis, etc. should be obtained if available.

Future Studies

Optimization of conditions using a factorial design

Summary of Experimental Factors and Levels to be Optimized

		Experimental Factor Levels				
Experimental Factors	Units	1	2	3	4	5
NADP+	mM	0.1	0.5	1	2	4
Glucose-6-Phosphate	mM	0.1	1	2	3	4
Glucose-6-Phosphate Dehydrogenase	units	0.1	0.5	1	2	4
Androstenedione (substrate)	nM	10	25	50	100	500
Protein	mg/mL	0.01	0.02	0.1	0.5	1
Incubation Time	min	10	15	30	60	120

Future Studies

Determination of variance of the optimized assay

- Using the optimized conditions determined for each preparation, three technicians independently conduct the assay on three separate days
- The results are assessed for technician-to-technician and day-to-day variance.

Future Studies

Determination of IC_{50} for:

- aminoglutethimide (non-steroidal aromatase Inhibitor)
- 4-hydroxyandrostenedione (potent steroidal aromatase inhibitor
- chrysin (potent flavonoid)
- genistein (weak isoflavonoid)
- ketoconazole (weak imidazole anti-fungal)
- econazole (potent imidazole anti-fungal)
- atrazine (affects aromatase gene expression; no aromatase inhibition)
- bis-(2-ethylhexyl)phthlate (affects aromatase gene expression; no aromatase inhibition)
- nonylphenol (affects AR/ER; no aromatase inhibition)
- lindane (affects StAR and cholesterol metabolism; no aromatase inhibition)
- dibenz(a,h)anthracene

Acknowledgements

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